

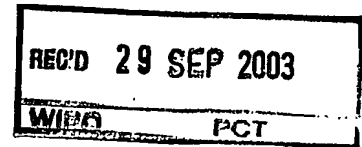
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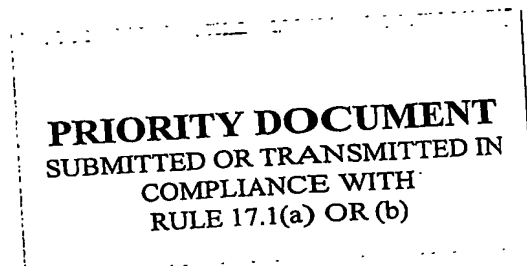
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Patentanmeldung Nr. Patent application No. Demande de brevet n°

02292254.6



Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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R C van Dijk



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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Infectious HCV pseudo-particles containing functional E1, E2 envelope proteins

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The invention relates to the generation and the use of HCV pseudo-particles containing native functional E1, E2 envelope glycoproteins assembled onto retroviral core particles. These particles are highly infectious and constitute a valid model of HCV virion.

5

World-wide several hundred millions of people are infected with hepatitis C virus (HCV) (Lavanchy et al., 1999). Progression to chronic disease occurs in the majority of HCV infected persons. Infection is associated with an increased risk for liver diseases and hepato-cellular carcinoma and has become the main indication for liver transplantation. HCV infection also increases the number of complications in HIV infected people (Dieterich, 2002). No vaccine is currently available to prevent new infections and the only treatment for chronic hepatitis C is interferon- α therapy, either alone or in combination with the guanosine analogue ribavirin. However, only ~40% of patients respond to treatment. Clearly, novel therapeutic strategies are urgently required as the health costs for HCV infected people are predicted to spiral dramatically in the next few decades.

HCV proteins structural and non structural proteins are expressed from a single polyprotein precursor and individually released in their respective cell compartments upon cleavage by cellular and viral proteases (Lindenbach and Rice, 2001). By analogy with other members of the Flaviviridae, HCV genomic organization suggests a virus consisting of a nucleocapsid comprising a viral genome and core protein (C) coated by a lipid envelop containing the two envelope glycoproteins E1 and E2.

So far, the study of hepatitis C virus (HCV), has been hampered by the low level of HCV particles in infected patients and by the lack of available efficient and reliable culture system for amplifying the virus (Lindenbach and Rice, 2001). Recently, a model for HCV replication, based on the self-replication of engineered minigenomes in cell culture, has been established (Blight et al., 2000; Lohmann et al., 1999). Although very useful to study HCV genomic replication, this system does not support production of HCV particles (Pietschmann et al., 2002).

Production of HCV virus-like particles (VLP) in insect cells has already been reported (WO 98/21338, Wellnitz et al., 2002; Baumert et al.; 1998, Owsianka et al., 2001). However these particles were not secreted and their extraction from intracellular compartments yielded VLP preparations that were not infectious.

Pseudotyped Vesicular Stomatitis Virus (VSV) viral particles have also been engineered with chimeric E1 and/or E2 glycoproteins whose transmembrane domains were modified to allow their transport to the cell surface (Buonocore et al., 2002; Matsuura et al., 2001). However, such modifications are likely to disturb

5 conformation and functions of the E1E2 complexes (Matsuura et al., 2001) and although such pseudo-particles stand among candidate HCV vaccines (Rose et al., 2001), their use as a tool to investigate HCV assembly and cell entry remains controversial (Buonocore et al., 2002). Chimeric HCV-BVDV (Bovine Viral Diarrhea Virus) particles were shown to be infectious for the human hepatocyte line Huh-7
10 (WO 00/75352). However, infectivity could not be neutralised by an anti-serum against HCV which indicated that these particles did not constitute a valid model of HCV virion.

New approaches are therefore sorely needed to study HCV assembly and cell entry in order to design HCV cell entry inhibitors and to study the humoral immune
15 response against HCV. Availability of infectious, amplifiable HCV particles would also provide useful material for the development of diagnostic application as well as therapeutical drugs.

The invention thus overcomes these hurdles by proposing infectious, HCV pseudo-particles harboring unmodified E1 and E2 glycoproteins.

Definitions

The terms "*vector*", "*cloning vector*" and "*expression vector*" mean the vehicle by which a DNA or RNA sequence (e.g. a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (e.g. transcription and
25 translation) of the introduced sequence. Vectors typically comprise the DNA of a transmissible agent, into which foreign DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. Generally, foreign DNA is inserted at one or more restriction
30 sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "*DNA construct*". A common type of vector is a "*plasmid*", which generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional

(foreign) DNA and which can readily be introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. Coding DNA is a DNA sequence that encodes a particular amino acid sequence for a particular protein or enzyme.

5 Promoter DNA is a DNA sequence which initiates, regulates, or otherwise mediates or controls the expression of the coding DNA. Promoter DNA and coding DNA may be from the same gene or from different genes, and may be from the same or different organisms. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and
10 prokaryotic hosts.

A "*coding sequence*" or a sequence "*encoding*" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, i.e., the nucleotide sequence encodes an amino acid sequence for that polypeptide,
15 protein or enzyme.

The term "*transfection*" means the introduction of a foreign nucleic acid (DNA, cDNA or RNA) into a cell so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein coded by the introduced gene or sequence. The introduced gene may include regulatory or control
20 sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell's genetic machinery. A host cell that receives and expresses introduced DNA or RNA has been "*transformed*".

The term "*host cell*" means any cell of any organism that is selected, modified, transformed, grown, or used or manipulated in any way, for the production of a
25 substance by the cell, for example the expression by the cell of a gene, a DNA sequence, a protein, a virion. In the context of the invention, the host cell is a mammalian cell. Suitable host cells include Huh-7 human hepatocellular carcinoma (Nakabayashi et al., 1982), Hep3B human hepatocellular carcinoma (ATCC HB-8064), HepG2 human hepatocellular carcinoma (HB-8065), HT-1080 human
30 fibrosarcoma (CCL-121), 293T human embryo kidney cells (ATCC CRL-1573), TE671 human rhabdomyosarcoma (ATCC CRL-8805), Jurkat human T cell leukemia (TIB-152), CEM human lymphoblastic leukemia (CCL-119), COS-7 African green monkey fibroblasts kidney (CRL-1651), VERO African green monkey kidney (CCL-81), PG-4 feline astrocyte (CRL-2032), BHK-21 golden hamster kidney (CCL-10),

CHO Chinese hamster ovary (ATCC CCL-61), and NIH3T3 mouse fibroblasts. In a specific embodiment said host cell is 293T.

As used herein, the term "*permissive cell*" is meant for a cell that is permissive for HCV infection.

5 "*Hepatitis C Virus*" or "*HCV*" is a member of the flavivirus family. HCV genome encodes a single polyprotein NH₂-C-E1-E2-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH that is processed co and post-translationally into both structural (N-terminal nucleocapsid protein termed "Core" (C), and glycoproteins E1 and E2) and non-structural (NS) proteins. In the context of the invention, HCV is of any genotype, e.g.
10 1, 2, 3, 4, 5, 6, and subtype, e.g. a, b, c, d, e, f, g, h, k, and variants thereof.

The term "*variant*" refers to the homologous polynucleotide sequences and corresponding amino acid sequences found in the different HCV strains owing to HCV hypervariability.

Preferably, HCV has either 1a or 1b genotype (Dubuisson *et al.*, 1994) that
15 stand among HCV genotypes that are the most prevalent and the most resistant to interferon- α therapy (Zein, 2000).

The term "*HCV-like particles*" or "*HCV pseudo-particles*" as used herein refers to non naturally occurring viral particles that comprise an envelope protein of HCV. The HCV pseudo-particles of the invention are infectious for a target cell. The
20 particles of the invention more particularly comprise retroviral core proteins. Such particles may be readily produced by one skilled in genetic engineering techniques. One can for instance refer to EP 1 201 750 that describes production of synthetic retroviral particles expressing an antigen for modulating an immune response.

In the context of the invention, the term "*infectious*" is used to describe the
25 capacity of the particles of the invention to complete the initial steps of viral cycle that lead to cell entry. However, upon interaction with the host cell, HCV-like particles may or may not produce progeny viruses.

The term "*an envelope protein of HCV*" denotes the E1 or E2 glycoprotein of HCV, or a mutant thereof.

30 By an "*E1 glycoprotein*" or "*E1 protein*" is meant a native envelope 1 protein (E1) from any genotype, subtype and variant of HCV strains.

By an "*E2 glycoprotein*" or "*E2 protein*" is meant a native envelope 2 protein (E2) from any genotype subtype and variant of HCV strains.

Preferably, E1 and E2 glycoproteins are derived from a same HCV strain.

The term "*mutant*" or "*mutation*" is meant for alteration of the DNA sequence that result in a modification of the amino acid sequence of native E1 or E2 proteins. Such a modification can be for instance the substitution and/or deletion of one or more amino acids. Mutants notably include fragments of native E1 and E2 proteins.

5 Variants are particular examples of naturally occurring mutants. Mutants are more particularly contemplated as useful for identifying the structural elements of E1 and/or E2 proteins necessary for maintaining cell infectivity or for increasing E1 and/or E2 antigenicity for vaccination purposes. In a preferred embodiment, the mutants encompass E2 glycoproteins wherein hypervariable region I has been deleted, while
10 the particles produced therefrom remain infectious.

As used herein, the term "*HCV core*" denotes a native core protein of the various HCV strains, a fragment thereof, or a variant thereof. The HCV core provides a signal peptide for the E1 or optionally E2 linked thereto that allows protein translocation to the endoplasmic reticulum. HCV core signal peptide correspond to
15 the last 21 residues of the carboxy-terminus of HCV core (GCSFSIFLLALLSCLTVPASA, SEQ ID N°1). According to an embodiment, the core protein is thus a N-terminally truncated form of HCV core (Δ C). Preferably Δ C comprises the last 21 residues of the carboxy-terminus of HCV core. In particular, HCV core may consist in the last 60 residues of the carboxy-terminus of HCV core.

20 In the context of the invention, the terms "*native*" or "*unmodified*" are indifferently used to describe a wild-type, full-length protein.

The term "*polyprotein*" as used herein is used to describe a protein construct made up of individual proteins that are joined together in a sequence whereby they retain their original relevant biological activities.

25 The term "*a polyprotein comprising a HCV core protein linked to HCV E1 protein and/or HCV E2 protein*" includes the CE1E2, CE2E1, CE1, CE2, Δ CE1E2, Δ CE1, and Δ CE2 polyproteins. "*CE1E2*" denotes a polyprotein comprising successively a HCV core protein, a HCV E1 protein and a HCV E2 protein. "*CE2E1*" denotes a polyprotein comprising successively a HCV core protein, a HCV E2 protein
30 and a HCV E1 protein. "*CE1*" denotes a polyprotein comprising a HCV core protein linked to a HCV E1 protein. "*CE2*" denotes a polyprotein comprising a HCV core protein linked to a HCV E2 protein. " *Δ CE1E2*" denotes a polyprotein comprising a carboxy terminus of HCV core protein, and HCV E1 and HCV E2 proteins. " *Δ CE1*" denotes a polyprotein comprising a carboxy terminus of HCV core protein, and a

HCV E1 protein. " $\Delta CE2$ " denotes a polyprotein comprising a carboxy terminus of HCV core protein, and a HCV E2 protein. $\Delta CE1E2$, as well as $\Delta CE2$, have been built by inserting a stop codon at the end of E2, whereas $\Delta CE1$ has been built by inserting a stop codon at the end of E1.

By "*retrovirus*" is meant a virus whose genome consists of a RNA molecule and that comprises a reverse-transcriptase, *i.e.* a member of the Retroviridae family. Retroviruses are divided into Oncovirus, Lentivirus and Spumavirus. Preferably said retrovirus is an oncovirus, *e.g.* MLV, ALV, RSV, or MPMV, a lentivirus, *e.g.* HIV-1, HIV-2, SIV, EIAV, or CAEV, or a spumavirus such as HFV. Genomes of these retroviruses are readily available in databanks.

In the context of the invention "*a nucleic sequence comprising a packaging competent retrovirus-derived genome*" is intended for a sequence that comprises the retroviral nucleic acid sequences known as "cis-acting" sequences. These include the Long Terminal Repeats (LTRs) for the control of transcription and integration, the psi sequence necessary for encapsidation, and the Primer Binding site (PBS) and polypurine track (PPT) sequences necessary for reverse transcription of the retroviral genome. Advantageously, said nucleic acid sequence comprising a packaging competent retrovirus-derived genome further comprises a transgene.

Said retroviral genome may be replication-defective or replication-competent, in the absence of any trans-complementing function. A replication-competent genome would further comprise the gag, pol, and env retroviral genes. In a replication-defective genome, the viral genes gag, pol, and env are deleted. However, assembly of viral pseudo-particles may be achieved by providing another vector that comprises gag, pol and env but that is defective for the "cis" sequences. Their expression allows the encapsidation of the transgene, excluding the genes necessary for the multiplication of the viral genome and for the formation of complete viral particles.

As used herein, the term "transgene" designates the gene that is expressed in the target cell upon infection by the particles of the invention.

Examples of transgenes include a gene encoding a molecule of therapeutic interest, a marker gene, a gene coding for an immune modulator, an antigen, or a suicide gene.

A "*marker gene*" denotes a gene whose expression is detectable. For instance marker gene expression can generate a detectable signal, such as a fluorescence

emission, a chromogenic reaction, or confer a growth advantage to the cells wherein it is expressed (antibiotic resistance genes).

An "*immune modulator*" refers to the product of a gene that modifies the activity of the immune system of a subject *in vivo*. Examples of immune modulators include cytokines, (e.g. interleukins, interferons, or haematopoietic colony stimulating factors), chemokines, and the like. Expression of an immune modulator by transformed cells may change the cellular environment and alter differentiation of immune cells and thus modify the type and the strength of immune response elicited against a given antigen.

An "*antigen*" refers to a molecule, such as a peptide, a polypeptide or a protein, against which an immune response is sought. Said antigen may be for instance a tumor, a bacterial, a pathogenic, a proteic, or a viral antigen.

A "*suicide gene*" is meant for a gene whose expression in cells induces programmed-cell death (apoptosis) such as the conditional Herpes Simplex virus type I thymidine kinase gene.

The "*core protein from a retrovirus*" refers to proteins encoded by the gag and pol genes. The gag gene encodes a polyprotein which is further processed by the retroviral protease into structural proteins that comprise the core. The pol gene encodes the retroviral protease, reverse-transcriptase, and integrase.

A "*pharmaceutically acceptable carrier*" refers to any vehicle wherein the vaccine composition according to the invention may be formulated. It includes a saline solution such as phosphate buffer saline. In general, a diluent or carrier is selected on the basis of the mode and route of administration, and standard pharmaceutical practice.

in the context of the present application, "*vaccination*" is intended for prophylactic or therapeutical vaccination. "*Therapeutical vaccination*" is meant for vaccination of a patient with HCV infection.

According to the invention, the term "*subject*" or "*patient*" is meant for any mammal likely to be infected with HCV. Human, chimpanzee, tamarin, and mice, especially human liver-xenografted mice are examples of hosts for HCV.

Production of HCV particles

The inventors have generated infectious pseudo-particles that contain functional, and more particularly unmodified, HCV glycoproteins assembled onto

retroviral core particles. HCV E1E2 were expressed from a polyprotein containing the core (C) protein or a fragment thereof, in particular the carboxy-terminus of the C protein, which served as signal peptide for E1 or E2, and the E1 and/or E2 glycoproteins.

5 The invention thus provides a method for producing hepatitis C virus (HCV)-like particles *ex vivo* comprising the steps of:

- providing a first nucleic acid sequence comprising a packaging competent retrovirus-derived genome;

- providing a second nucleic acid sequence comprising a cDNA encoding the
10 core proteins from said retrovirus;

- providing a third nucleic acid sequence comprising a cDNA encoding a polyprotein comprising successively a HCV core protein, and a HCV E1 protein and/or a HCV E2 protein;

- transfecting host cells with said nucleic acid sequences and maintaining the
15 transfected cells in culture for sufficient time to allow expression of the cDNAs to produce structural proteins from hepatitis C virus and retrovirus; and allowing the structural proteins to form virus-like particles.

The invention further provides a method for producing hepatitis C-virus (HCV)-like particles *in vivo*, which method comprises the steps of :

- 20 - providing a first nucleic acid sequence comprising a packaging competent retrovirus-derived genome;

- providing a second nucleic acid sequence comprising a cDNA encoding the core proteins from said retrovirus;

- providing a third nucleic acid sequence comprising a cDNA encoding a
25 polyprotein comprising successively a HCV core protein, and a HCV E1 protein and/or a HCV E2 protein;

- transfecting cells of a subject *in vivo* with said nucleic acid sequences, to allow expression of the cDNAs to produce structural proteins from hepatitis C virus and retrovirus; and to allow the structural proteins to form virus-like particles.

30 Another aspect of the invention is the use of three nucleic acid sequences for the preparation of a medicament useful as a vaccine against hepatitis C, wherein the nucleic acid sequences are :

- a first nucleic acid sequence comprising a packaging competent retroviral-
derived genome;

- a second nucleic acid sequence comprising a cDNA encoding core proteins from said retrovirus;

- a third nucleic acid sequence comprising a cDNA encoding a polyprotein comprising successively a HCV core protein, and a HCV E1 protein and/or a HCV E2 protein ;

and, when transferred into cells of a subject, the nucleic acid sequences allow the production of structural proteins from hepatitis C virus and retrovirus, wherein the structural proteins form virus-like particles that are immunogenic.

For the purpose of transfection, said first, second and third nucleic acid sequences may be carried on a same vector, or on two or three separated vectors.

An example of HCV E1E2 and retroviral expression constructs is shown in figures 6A and 6B.

According to a specific embodiment, said packaging competent retroviral genome and core proteins are derived from a retrovirus selected from the group consisting of MLV, ALV, RSV, MPMV, HIV-1, HIV-2, SIV, EIAV, CAEV, and HFV.

Advantageously, the packaging competent retroviral genome further comprises a marker gene or an immune modulator.

In the method of the invention, said polyprotein may comprise a HCV core protein linked to a HCV E1 protein, a HCV core protein linked to a HCV E2 protein, or successively a HCV core protein, a HCV E1 protein and a HCV E2 protein, or successively a HCV core protein, a HCV E2 protein and a HCV E1 protein.

According to an embodiment, E1 and/or E2 are native proteins. According to another embodiment, E1 and/or E2 glycoproteins are mutated to obtain particles that are useful for characterizing the glycoprotein determinants for HCV infectivity.

Preferably, said E1 and E2 glycoproteins are both derived from a same HCV strain.

According to another embodiment said HCV core protein is a carboxy terminus form (ΔC) of HCV core protein. In particular, said HCV core protein may comprise the last 21 amino acids of the carboxy-terminus of HCV core.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al., 1989 ; DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985) ; Oligonucleotide Synthesis (M.J. Gait ed. 1984) ; Nucleic Acid Hybridization

[B.D. Hames & S.J. Higgins eds. (1985)] ; Transcription and Translation [B.D. Hames & S.J. Higgins, eds. (1984)] ; Animal Cell Culture [R.I. Freshney, ed. (1986)] ; Immobilized Cells and Enzymes [IRL Press, (1986)] ; B. Perbal, A Practical Guide To Molecular Cloning (1984) ; F.M. Ausubel et al., 1994.

5 In particular, the vectors of the invention may be introduced into the target cell by means of any technique known for the delivery of nucleic acids to the nucleus of cells, either in culture, *ex vivo*, or *in vivo*.

 Introduction of the nucleic acid sequences may be performed by any standard method well known by one skilled in the art, e.g. transfection, 10 electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, or use of a gene gun (see for instance Wu et al., 1992 ; Wu et al, 1988).

 The donor nucleic acid targeting system can also be introduced by lipofection. In certain embodiments, the use of liposomes and/or nanoparticles is 15 contemplated for the introduction of the donor nucleic acid targeting system into host cells. Nanocapsules can generally entrap compounds in a stable and reproducible way. Ultrafine particles (sized around 0.1 μm) that can be designed using biodegradable polyalkyl-cyanoacrylate polymers are contemplated for use in the present invention, and such particles may be easily made.

20 Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 μm . Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution 25 in the core. The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes (Felgner et al., 1989).

In vivo targeted gene delivery is described in international patent publication WO 95/28 494. Alternatively, the vector can be introduced *in vivo* by lipofection, 30 using liposomes or nanoparticles as above described. It is also possible to introduce the vector *in vivo* using techniques that are similar to the techniques that are employed *in vitro* (e.g. transfection, electroporation...).

HCV-like particles

In the method described above no structural modifications of the E1E2 glycoproteins are required for their correct assembly on retroviral cores. The method of the invention thus makes it possible to generate high titre infectious HCV pseudo-
5 particles with functional E1E2 glycoproteins. As demonstrated herein, these particles constitute a valid model of HCV virion as regards to early steps of viral infection cycle.

The invention further relates to an infectious HCV-like particle, comprising the core proteins from a retrovirus, and E1 and/or E2 HCV glycoprotein(s). Such a
10 particle is obtainable by a method as described above.

According to an embodiment, the infectious particle of the invention may comprise native HCV E1 protein, native HCV E2 protein, or native HCV E1 protein and native HCV E2 protein. Preferably said E1 and E2 glycoproteins are both derived from a same HCV strain. According to another embodiment, E1 and/or E2
15 glycoproteins are mutated.

In a particular embodiment, the hypervariable region I (located in the N-terminus region, *i.e.* the first 27 aminoacids of the E2 protein after the signal peptide) is deleted. The HCV-like particles (called Δ HVR1) produced with E2 protein deleted from this region are particularly advantageous as a diagnostic tool or in vaccination,
20 to enhance induction or binding of neutralizing antibodies. The Δ HVR1 HCV-like particles are also of interest to identify the epitopes/mechanisms within the HCV glycoproteins that can be targeted/inhibited by neutralizing antibodies or other therapeutic agents.

Said retrovirus may be selected from the group consisting of MLV, ALV, RSV, MPMV, HIV-1, HIV-2, SIV, EIAV, CAEV, and HFV.
25

Advantageously, said infectious particles further carry a transgene. For instance said transgene may be a marker gene which make it possible to follow-up cell infection by the infectious particles of the invention and can find application for instance in the identification of a cell receptor involved in HCV entry. Said transgene
30 can also be a gene encoding a molecule of therapeutic interest and/or a suicide gene. Accordingly, the particles of the invention that specifically target primary or cancerous hepatocytes comprise a useful vector for gene transfer and/or gene therapy.

Use of the infectious HCV-like particles of the invention

High infectivity of these particles makes it possible for the investigation of the role of HCV E1 and E2 glycoproteins and their potential receptors in cell entry, HCV host-range and neutralisation by antibodies from HCV patient sera.

5 The invention therefore concerns the use of an HCV-like infectious particle as described above, for *ex vivo* identification of a cell receptor for HCV E1 and/or E2 glycoprotein.

 According to an embodiment, the invention provides a method for *ex vivo* identification of a receptor for HCV E1 and/or E2 glycoprotein comprising detection of
10 the binding of said particle to a cell receptor. More specifically, the method may comprise the steps consisting of:

- contacting a cell susceptible to HCV infection with an infectious HCV-like particle of the invention, under conditions sufficient to allow specific binding of said particle to a receptor expressed at the surface of said cell;
- 15 - detecting binding of said particle to a receptor; and
- identifying said receptor.

 A cell susceptible to HCV infection may preferably be selected from the group consisting of a hepatocyte cell line, such as Huh-7 human hepatocellular carcinoma (Nakabayashi et al., 1982), Hep3B human hepatocellular carcinoma (ATCC HB-
20 8064), or HepG2 human hepatocellular carcinoma (HB-8065), and a primary human hepatocyte. Primary human hepatocytes may be isolated from human adult biopsy samples according to procedures well-known by one skilled in the art. One can for example refer to Guguen-Guillouzo and Guillouzo (1986). Otherwise such cells are commercially available, and can be purchased for instance from Biopredic
25 International (Rennes, France).

 Detection of particle binding to a receptor can be achieved according to classical procedures well-known by one skilled in the art. For instance, this could involve radioactive, enzyme or fluorescent labelling of the particles of the invention, and subsequent detection with an appropriate method. A number of fluorescent
30 materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red. Enzyme labels consist in conjugation of an enzyme to a molecule of interest, e.g. a polypeptide, and can be detected by any of colorimetric, spectrophotometric, or fluorospectrophotometric techniques. Flow

cytometry analysis (FACS) together with labelled antibodies directed against E1 or E2 proteins harboured by the pseudo-particles of the invention is also appropriate.

According to another embodiment, the invention provides a method for *ex vivo* identifying a cell receptor for HCV comprising the step consisting of:

- 5 - transfecting a cell which is not permissive for HCV infection with a nucleic acid sequence encoding a protein likely to be a receptor for HCV;
- contacting said transformed cell with a HCV-like particle of the invention;
- determining whether said transformed cell has become permissive or not for HCV infection; and
- 10 - identifying as a cell receptor for HCV said protein expressed by the transformed cell that has become permissive.

Determination of whether the transformed cell has become permissive for HCV infection can be readily achieved using the HCV-like particles of the invention. In particular, where said particles carry a marker gene, such as GFP, permissivity
15 (i.e. the capacity of cells to be infected with HCV, and in particular with HCV-like particles) can be assessed by FACS analysis of the transformed cells. Where the marker gene is an antibiotic resistance gene, identification of cells infected by HCV-like particle is readily achieved through exposure to said antibiotic.

Where one do not suspect a given protein to be a receptor for HCV entry in
20 cells, the above method can advantageously be adapted for the screening and the identification of a cell receptor for HCV. In particular, an expression cDNA library can be prepared, for instance from a cDNA library obtained by reverse-transcription of cellular mRNAs from a cell permissive for HCV infection. Expression of such a cDNA library would be driven by a constitutive promoter whose nucleic acid sequence has
25 been fused to the cDNA library in suitable vectors. Such a library would contain a vector encoding a cell receptor for HCV. Non permissive cells can then be transfected with this expression library and further screened for the identification of a cell receptor for HCV.

To this end, the invention proposes a method for *ex vivo* identifying a cell
30 receptor for HCV comprising the step consisting of:

- providing an expression cDNA library obtained from a cell permissive for HCV infection;
- transfecting cells that are not permissive for HCV infection with said expression cDNA library;

- contacting said transformed cells with HCV-like particles of the invention;
- identifying and isolating those transformed cells that have become permissive for HCV infection;

- isolating the expression vector transfected in cells that have become

5 permissive; and

- identifying as a receptor for HCV the proteins encoded by the cDNA sequence of said isolated expression vectors.

Determination of whether the transformed cell has become permissive for HCV infection can be readily achieved using the HCV-like particles of the invention.

10 In particular, where said particles carry a marker gene, such as GFP, permissivity (*i.e.* the capacity of cells to be infected with HCV, and in particular with HCV-like particles) can be assessed by FACS analysis of the transformed cells. Where the marker gene is an antibiotic resistance gene, identification of cells infected by HCV-like particle is readily achieved through exposure to said antibiotic.

15 Advantageously, the expression cDNA library is expressed from retroviral vectors that comprises glycoproteins that allow infection of the HCV non permissive cells. Such glycoproteins can be the VSV-G glycoprotein derived from vesicular stomatitis virus (VSV) whose receptor is expressed in most cell types *ex vivo*. Such viral particles can be assembled using a packaging competent retrovirus-derived

20 genome that comprises the expression cDNA library, and optionally a marker gene. According to this embodiment the method for isolating the expression vector expressed in cells that have become permissive to infection by the HCV-like particles of the invention is greatly facilitated. Indeed this latter embodiment is particularly advantageous in that the process of cell infection with retroviral vectors has greater

25 efficacy, as compared to cell transfection. Furthermore, cell infection leads to stable integration of viral genome in the cellular genome. Accordingly, transgenes, *i.e.* cDNA and marker gene that are carried by the pseudo-particles of the invention, are found to be stably expressed by infected cells. This in contrast with classical vectors used for transfection that do not integrate into cellular genome and for which

30 expression may be transient.

In another aspect, the invention relates to the use of an infectious particle as defined above, for the identification of molecules capable of interfering with HCV entry in cells. In particular, herein is provided a method of *ex vivo* screening or

identification of molecules capable of interfering with HCV entry in cells comprising

comparison of the level of cell infection by the particles of the invention in the presence or the absence of a candidate molecule. Said method preferably comprises the steps consisting of:

- contacting a cell susceptible to HCV infection with an infectious HCV-like particle, in the absence or presence of a candidate molecule, under conditions that allow cell infection with HCV-like particle in the absence of any candidate molecule;
- assessing cell infectivity in the absence and in the presence of said candidate molecule;
- comparing cell infectivity measured in presence of said candidate molecule with cell infectivity measured in absence of any candidate molecule;
- identifying as a molecule capable of interfering with HCV entry the candidate molecule for which cell infectivity, as measured in the presence of said molecule, is decreased as compared to cell infectivity measured in the absence of any candidate molecule.

Contacting a cell susceptible to HCV infection with an infectious HCV-like particle, and a candidate molecule can be carried out by contacting simultaneously said cell, HCV-like particle and candidate molecule. Otherwise, two of these three elements can be contacted under conditions sufficient to allow their interaction before addition of the third missing element.

Cell infectivity can be readily assessed by one skilled in the art. One can take advantage of the embodiment wherein the infectious HCV-like particle carries a detectable marker gene to detect cell infection. In a preferred embodiment, the marker gene is a fluorescent marker gene, such as GFP, and the infection is detected by means of fluorescence measurement, for instance by flow cytometry analysis of cells contacted with said infectious particles.

A cell suitable to be used in the method of identification of molecules interfering with HCV cell entry may be selected from the group consisting of a hepatocyte cell line and a primary human hepatocyte, as described above.

Such molecules capable of interfering with HCV entry in cells may constitute new antiviral drugs.

The infectious particles of the invention are further useful for diagnosis of HCV infection and follow-up of HCV infection, for instance to assess efficacy of a therapy in a patient.

The invention thus concerns the use of an infectious HCV-like particle for the *in vitro* detection of antibodies directed against HCV in a biological sample from a subject susceptible to be infected with HCV. Said biological sample may be a biological fluid, such as blood or serum, or a tissue biopsy, for instance a tissue sample from liver biopsy. In a specific embodiment, said antibodies are directed against E1 and/or E2 HCV glycoproteins.

Accordingly, the invention provides a method of *in vitro* diagnosis of a HCV infection in a patient comprising detecting immune complexes formed by interaction of anti-HCV antibodies likely to be present in a biological sample of the patient, with HCV-like particle of the invention. Said method may in particular comprise the steps consisting of:

- contacting a biological sample with an infectious HCV-like particle of the invention under conditions sufficient to allow formation of complexes by binding of said infectious particle to antibodies directed against HCV present in the biological sample;

- detecting said complexes, which presence is indicative of a HCV infection.

The presence of antibodies reactive with HCV-like particles can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the HCV-like particle and the antibody or antibodies reacted therewith.

In another embodiment, said method of *in vitro* diagnosis of a HCV infection in a patient comprises detecting an inhibitory effect of anti-HCV antibodies likely to be present in a biological sample of the patient, on the infection of a permissive cell by a HCV-like particle of the invention. Said method may in particular comprise the steps consisting of:

- contacting a cell permissive for HCV infection with a HCV-like particle and a biological sample;

- comparing cell infectivity measured in presence of said biological sample with cell infectivity measured in absence of said biological sample;

- detecting the inhibition of HCV-like particle infection of a permissive cell as a decrease in cell infectivity measured in presence of said biological sample compared with cell infectivity measured in absence of said biological sample, said inhibition being indicative of a HCV infection.

This embodiment is advantageous in that the method relies on the detection of the specific antibodies that are neutralizing for cell infection, that is those patient's antibodies that are effective against viraemia.

In a further embodiment of this invention, commercial diagnostic kits may be useful to carry out the above diagnosis methods, by detecting the presence or absence of immune complexes formed by HCV particles and antibodies directed against HCV in a biological sample from a subject susceptible to be infected with HCV, or by detecting an inhibition of HCV-like particle infection of a permissive cell by anti-HCV neutralizing antibodies likely to be present in a biological sample of the patient. Such kits may comprise at least a HCV-like particle of the present invention. Where the method involves detection of immune complexes, the kits may further comprise appropriate means of detection of said immune complexes. Preferably the kit of the invention further comprises directions, and protocols, depending upon the method selected, e.g., "competitive", "sandwich", and the like. The kits may also contain peripheral reagents such as buffers, stabilizers, etc..

In another aspect of the invention, the infectious HCV-like particles may be used for vaccination purposes.

According to an embodiment, the invention thus proposes a method of vaccination, notably against HCV infection, that comprises administration of a HCV-like particle to a subject in need thereof. The invention also relates to a vaccine composition comprising a HCV-like particle and a pharmaceutically acceptable carrier. The invention further provides a immunogenic composition comprising in a pharmaceutical acceptable carrier, a HCV-like particle disclosed herein.

The vaccine and immunogenic compositions of the invention may be drawn to confer immunity, or elicit an immune response against HCV.

However, where the HCV-like particles of the invention further carry an additional gene encoding another antigen, different from HCV antigens, the invention

provides a recombinant viral vaccine useful to raise an immune response against said antigen. Actually, the use of pseudo-particles described herein makes it possible to improve the elicited immune response through combining several presentation and processing pathways of an antigen. For instance, a vaccine composition of the

invention, when administered, results in the HCV-like particles infecting cells of the host. The transgene encoding the antigen is then integrated in the cellular genome, and subsequently expressed by the cell, such that there is both a cellular and a humoral immune response elicited by the vaccine composition.

Advantageously, the HCV-like particles may further carry a transgene encoding an immune modulator, which allows for enhancement of the raised immune reaction.

The vaccination or immunogenic composition of the present invention may additionally contain an adjuvant. A number of adjuvants are known to those skilled in the art. Examples of suitable adjuvants include, for example, include aluminum hydroxide; Saponin; detergents such as Tween 80; animal, mineral or vegetable oils, Corynebacterium or Propionibacterium -derived adjuvants; Mycobacterium bovis (Bacillus Calmette and Guérin, or BCG); cytokines; acrylic acid polymers such as carbomer; EMA; or combinations thereof.

The route of administration is any conventional route used in the vaccine field. As general guidance, a vaccine composition of the invention is administered via a mucosal surface, e.g., an ocular, intranasal, pulmonary, oral, intestinal, rectal, vaginal, and urinary tract surface; or via a parenteral route, e.g., by an intravenous, subcutaneous, intraperitoneal, intradermal, intraepidermal, or intramuscular route. The choice of administration route depends on the formulation that is selected.

In still another embodiment the particles of the invention may be used as vectors for gene transfer and/or gene therapy. Gene therapy is defined as the introduction of genetic material into a cell in order to either change its phenotype or genotype. Owing to their tropism for hepatic cells, either primary or cancerous, the HCV-like particles described herein comprise an efficient gene delivery system specific for hepatocytes. Furthermore, such a delivery system is amenable to scale up for reproducibly producing large titers of infectious, replication-defective HCV-like particles.

Accordingly, the invention relates to a method for *in vivo* or *in vitro* transferring a transgene of interest in a cell, which method comprises infecting a cell with a HCV-like particle of the invention, wherein the particle carries a transgene of interest.

The invention further relates to the use of a HCV-like particle of the invention, that carries a transgene of interest, for the preparation of a medicament for the prevention or treatment of a disease in a patient, wherein the HCV-like particle allows the transfer of the transgene of interest into a cell of the patient, and encodes a product that has a prophylactic or therapeutic effect against the disease.

Preferably, the targeted cell is a hepatic cell.

The invention will be further understood in view of the following examples and the annexed figures.

LEGEND TO THE FIGURES :

Figure 1 depicts the results of infectivity experiments performed with HCV pseudo-particles of 1a genotype and different target cell types. Results are displayed as transducing units (TU) per ml of supernatant (mean \pm standard deviations of up to six experiments) for HCVpp. The infectivity on Huh-7 cells of HCVpp concentrated 100 times by ultra-centrifugation is shown (100x).

Figure 2 represents the infectious titres determined on Huh-7 target cells with HCV pseudo-particles generated without E1E2 (lane a), without retroviral core proteins (lane b), with MLV-G2A assembly-defective core proteins (lane c), with HIV-1 core proteins (lane d), with E1 or E2 alone (lane e or f, respectively), with E1+E2 expressed in *trans* from two independent vectors (lane g), with HCV-1a E1E2 expressed from the same vector in *cis* (lane h), or with HCV-1b E1E2 (lane i). HCVpp were treated with 25 μ M AZT (3'-azido-3'-deoxythymidine; Sigma-Aldrich, France) before and during infection of target cells (lane j). Results are expressed as TU/ml and are displayed as mean \pm standard deviations of up to four experiments.

Figure 3 shows the results of infection on human primary hepatocytes derived from two donors with of HCVpp of genotype 1a. Infectivity is expressed as percentage of infectivity determined on Huh-7 cells.

Figure 4 displays neutralisation of HCV pseudo-particles with monoclonal antibodies against E1 (A4) or E2 (H31, H33, H35, H44, H48, H53, H54, H60 and

H61) glycoproteins of genotype 1a, with pooled antibodies (Hmix), with no antibodies or using pseudo-particles generated with VSV-G (control). Neutralization of the control was achieved with the VSV-G neutralising 41A.1 monoclonal antibody.

Results are expressed as the percentages of inhibition of the average infectious titres

5 \pm standard deviations relative to incubation in the absence of antibodies.

Figure 5 represents neutralisation of HCVpp with HCV patient sera. The genotype of HCV diagnosed in these patients is indicated in brackets. Results are expressed as percentages of inhibition of the average infectious titres \pm standard deviations relative to incubation with control sera from healthy individuals.

10 Figures 6A and 6B show HCV E1E2 and retroviral expression constructs. (A) :

A cDNA derived from the HCV polyprotein gene was used to express the E1E2 glycoproteins and the carboxy-terminus of the C protein, which provides the signal peptide for E1 (SP E1). The position of stop codons (star) inserted in the expression constructs to terminate translation of the proteins is shown. The transmembrane

15 domain (TMD) of E1 provides the signal peptide (SP E2) for the E2 glycoprotein. (B) :

The expression constructs encoding the different components required to assemble infectious pseudo-particles are shown. The filled boxes represent the viral genes and the marker gene (GFP) transferred to the infected cells. The open boxes show the *cis*-acting sequences. LTR, long terminal repeat ; CMV, human cytomegalovirus

20 immediate-early promoter ; PBS, primer binding site ; ψ , packaging sequence ; PPT, poly-purine track ; polyA, polyadenylation site ; SD, splice donor site ; SA, splice

acceptor site. Vector particles were produced by cotransfection of plasmids harboring the packaging functions, the transfer vector and the viral glycoproteins into 293 T

cells. The viral glycoproteins were the HCV E1 and E2 glycoproteins, expressed

25 individually or as a CE1E2, the VSV-G or the RD114 glycoproteins. The supernatants of transfected cells were collected during transient expression, concentrated by ultracentrifugation, and used for target cell transduction.

Figure 7 shows incorporation of Δ HVR1 HCV pseudo-particles. Immunoblots of lysates of 293 T transfected cells and of pseudo-particles pelleted through 20 %
30 sucrose-cushions are shown. The positions of the molecular weight markers are shown (kDa).

Figure 8 represents the infectivity of Δ HVR1 HCV pseudo-particles. Supernatants from producer cells were diluted and used to infect Huh-7 target cells before assessing transduction efficiency three days post-infection by FACS analysis.

5 EXAMPLES :

EXAMPLE 1 : **Generation of HCV pseudo-particles (HCVpp)**

HCV pseudo-particles (HCVpp) were generated by assembling full-length, unmodified E1 and E2 glycoproteins onto retroviral core proteins derived from murine leukemia virus (MLV). To investigate further whether functional HCVpp could also be produced with E1 and E2 expressed in *trans* or with only one of the two glycoproteins, expression vectors that encoded individually either E1 or E2 glycoproteins were designed.

15 *Construction of expression vectors encoding the viral components, i.e., E1, E2, or E1E2 glycoproteins and viral core proteins*

Plasmids expressing wild type E1E2 polyproteins were constructed by standard methods (Sambrook et al., 1989).

Briefly, the pHCMV-7a expression vector encoding the E1 and E2 glycoproteins from a 1a type HCV was generated by inserting the blunted *Cla*I and *Stu*I restriction fragment encoding the last 60 residues of HCV core (C) and all of E1 and E2 proteins from the pTM1p5E1E2(745) vector (Op De Beeck et al., 2000) into the *Bam*HI digested and Klenow blunted vector pHCMV-G (Nègre et al., 2000).

The pHCMV-E1 expression vector, expressing only HCV E1 glycoprotein, was derived from pHCMV-7a by adding a stop codon to the C-terminus of E1 with primers 5'-actggacgacgcaaagctgc (SEQ ID N°2) and 5'-cgcggtacacctacgcgtcgacgccggcaaa (SEQ ID N°3). The resulting PCR fragment was digested with *Bam*HI and ligated into *Bam*HI-digested pHCMV-7a.

pHCMV-E2, expressing only HCV E2 glycoprotein, was obtained by fusing the N-terminus of E2 to the C-terminus of the HCV core using two PCR fragments generated with the two primer pairs 5'-tgcccgttcagccgaaaccacgtcaccggggga (SEQ ID N°4) + 5'-gccagaagtcagatgctcaagg (SEQ ID N°5) and 5'-tactctgagtccaaaccg (SEQ ID N°6) + 5'-gtgacgtgggttcggctgaagcgggcacagtcag (SEQ ID N°7). The two PCR

fragments were then fused in a second round PCR. The resulting DNA fragment was digested with *Bam*HI and ligated into *Bam*HI cut phCMV-7a.

The sequence of the phCMV-ΔC E1 vector is shown in SEQ ID No 8, whereas the aminoacid sequence of E1 protein is shown in SEQ ID No 9. The sequence of the phCMV-ΔC E1E2 vector is shown in SEQ ID No 10, whereas the aminoacid sequence of the E1E2 polyprotein is shown in SEQ ID No 11. The sequence of the phCMV-ΔC E2 vector is shown in SEQ ID No 12, whereas the aminoacid sequence of E2 protein is shown in SEQ ID No 13.

Expression vectors for E1E2 glycoproteins of 1b genotype were constructed by similar strategies.

HCV E1E2 were therefore expressed from a polyprotein containing the carboxy-terminus of the core (ΔC) protein, which served as signal peptide for E1, E2 or E1 and E2 glycoproteins.

Generation of HCV pseudo-particles

Retroviruses were chosen as platforms for assembly of HCVpp because their cores can incorporate a variety of different cellular and viral glycoproteins and because they can easily package and integrate genetic markers into host cell DNA.

HCVpp were produced by transfecting 293T human embryo kidney cells (ATCC CRL-1573) with three expression vectors encoding a ΔCE1E2, ΔCE1 or ΔCE2 polyprotein, the MLV core proteins and a packaging-competent MLV-derived genome harbouring the GFP (green fluorescent protein) marker gene. This construct contains the GFP marker gene, whose expression is driven by the CMV (cytomegalovirus) immediate early promoter. Both CMV and GFP nucleic acid sequences were inserted in a retroviral vector derived from MLV in which the gag, pol and env viral gene were removed and in which the retroviral cis-acting elements that control vector genome packaging, reverse transcription and integration were retained.

Control pseudo-particles were generated with the VSV-G glycoprotein (Nègre *et al.*, 2000), the RD114 virus envelope glycoprotein (Sandrin *et al.*, 2002), and/or with assembly-defective MLV core proteins (MLV-G2A) (Swanstrom *et al.*, 1997).

Briefly, expression constructs were transfected into 2.5×10^6 293T cells seeded the day before in 10 cm plates using a calcium-phosphate transfection protocol (Clontech, France) according to the manufacturer's recommendations. The medium

(8 ml/plate) was replaced 16 hrs after transfection. Supernatants containing the pseudo-particles were harvested 24 hrs later, filtered through 0.45µm-pore-sized membranes and processed as described before (Nègre *et al.*, 2000).

5 *Immunoblot analysis of structural components of the pseudo-particles*

Lysates of transfected cells and of pseudo-particles pelleted through 20% sucrose-cushions were immunoblotted. Expression of E1 and E2 glycoproteins from HCV-1a genotype and of MLV core proteins was revealed in reducing and denaturing conditions with monoclonal antibodies against E1 (A4) (Dubuisson *et al.*, 1994) and
10 E2 (H52) (Flint *et al.*, 1999) or with an anti-capsid (MLV CA) antiserum (ViroMed Biosafety Laboratories, USA), as described previously (Sandrin *et al.*, 2002). VSV-G expressed in control pseudo-particles was detected with the monoclonal antibody P5D4 (Sigma-Aldrich, France).

Analysis of immunoblots of transfected cells showed that the structural
15 components of the pseudo-particles were readily detected at the expected molecular weights; *i.e.* 30 kDa for E1, 60 kDa for E2, 60 kDa for VSV-G and 70 kDa for the RD114 glycoprotein. MLV core proteins were detected as a Gag protein precursor of 65 kDa that was partially processed by the MLV protease into mature core components.

20 The E1 and E2 glycoproteins were readily detected in the pellets of purified virions generated with the wild-type MLV core particles but not with the viral assembly-deficient MLV-G2A mutant. E2 present within the viral pellets migrated slightly slower than the cell-associated forms, due to modifications of the associated glycans by Golgi enzymes.

25 The presence of VSV-G in viral pellets generated with MLV-G2A assembly-defective core proteins is due to empty vesicles formed by VSV-G itself (Nègre *et al.*, 2000).

30 Comparison of the relative levels of VSV-G or HCV glycoproteins in producer cell lysates versus viral pellets suggests efficient incorporation of E1 and E2 into viral particles. Likewise, could E1 and E2 glycoproteins efficiently assemble on retroviral core proteins derived from HIV-1, raising the possibility of pathogenic interactions between the two parental viruses, *in vivo*, as co-infection of patients with HCV and HIV is prevalent (Dieterich, 2002).

Altogether, these results indicate that transient over-expression of E1 and E2 in 293T cells leads to specific and efficient incorporation of HCV glycoproteins into pseudo-particles generated with retroviral cores. Since HCV envelope glycoproteins

have been shown to be retained in the ER (Op De Beeck et al. 2001), this implies

that HCVpp form by budding into the ER lumen or, alternatively, that saturation/leakiness of the ER retention allows a fraction of E1E2 to reach the cell surface where MLV budding normally occurs (Swanstrom and Wills, 1997).

Individual expression or co-expression in *trans* of E1 and E2, from distinct expression units, led to normal levels of synthesis, as compared to expression of both glycoproteins in *cis*, from a single E1E2 polyprotein. Moreover, HCVpp were found to incorporate similar levels of E2 glycoprotein, whether it was expressed alone, or co-expressed in *cis* or in *trans* with E1. Finally, incorporation of E1 expressed alone or co-expressed in *trans*, with E2, occurred but at reduced levels, consistent with the chaperone activity of E2 (Op De Beeck et al., 2001). Formation of HCVpp carrying E1 or E2 only will allow investigation of their respective roles in HCV cell entry and infectivity.

EXAMPLE 2 : HCVpp cell line infectivity

Infectivity of HCVpp was assessed on a panel of target cell lines : Huh-7 human hepatocellular carcinoma (Nakabayashi et al., 1982), Hep3B human hepatocellular carcinoma (ATCC HB-8064), HepG2 human hepatocellular carcinoma (HB-8065), HT-1080 human fibrosarcoma (CCL-121), 293T, TE671 human rhabdomyosarcoma (ATCC CRL-8805), Jurkat human T cell leukemia (TIB-152), CEM human lymphoblastic leukemia (CCL-119), COS-7 African green monkey fibroblasts kidney (CRL-1651), VERO African green monkey kidney (CCL-81), PG-4 feline astrocyte (CRL-2032), BHK-21 golden hamster kidney (CCL-10), CHO Chinese hamster ovary (ATCC CCL-61), and NIH3T3 mouse fibroblasts. Target cells were grown as recommended by the ATCC.

Target cells (seeded the day before 8×10^4 cells/well in 12-well plates) were incubated for 3 hrs with dilutions of supernatants from producer cells containing the HCVpp, then washed and cultured until expression of the GFP marker gene harboured by the virions was assessed by FACS analysis 72 hrs later (Sandrin et al., 2002). Since the HCVpp were generated with replication-defective viral components,

this procedure allowed evaluation of the specific infectivity of the pseudo-particles after a one-round infection process.

Infectious titres of up to 1.1×10^5 TU/ml were detected for the HCVpp on Huh-7 human hepato-carcinoma cells (Figure 1). Upon concentration of the producer cell supernatants by ultra-centrifugation (Sandrin *et al.*, 2002), infectious titres of 2×10^6 TU/ml, on average, could be readily obtained. The other target cell types used in the infection assays displayed weaker (Hep3B, HepG2, HT1080, 293T, Vero) or undetectable (TE671, Jurkat, CEM, Cos-7, BHK-21, CHO, PG-4, NIH3T3) levels of infection with the HCVpp. The infectivity of control pseudo-particles generated with VSV-G ranged from 5×10^6 to 2×10^7 TU/ml depending on the target cell type indicating that all these cells were readily infected with the control pseudo-particles. This suggested that all the molecules necessary for HCV entry were specifically expressed in the former cell types only.

Infectious HCVpp could be generated at comparable efficiencies with E1E2 glycoproteins derived from HCV genotypes 1a and 1b (lanes h and i; Figure 2) and/or with retroviral core proteins derived from either HIV-1 or MLV (lanes d and h; Figure 2).

Incubation of HCVpp and target cells with AZT, a reverse-transcriptase inhibitor that prevents conversion of the retroviral RNA genome of the HCVpp as integration-competent proviral DNA, inhibited transduction (lane j; Figure 2).

Moreover, long-term expression of GFP could be demonstrated after serial passaging of the infected cells for more than one month.

These results indicated that infection of the target cells led to integration in host cell DNA and stable expression of the GFP marker gene transduced by the HCVpp. Additionally, no infection could be obtained with viral particles lacking both E1 and E2 glycoproteins, or lacking core proteins, or, alternatively, when using the MLV-G2A assembly-defective core proteins (lanes a-c; Figure 2).

Altogether, these results demonstrated that HCVpp harbouring the E1 and E2 glycoproteins and retroviral core proteins were infectious, leading to retroviral-mediated integration of their vector genome.

Finally, despite reduced levels of E1 incorporation, HCVpp generated with E1 and E2 glycoproteins expressed in *trans*, from distinct vectors, were nearly as infectious as those generated with the E1E2 polyprotein construct (lanes g and h; Figure 2). However, HCVpp assembled with either E1 or E2 glycoproteins only were

500-fold less infectious (lanes e-f; Figure 2), demonstrating that both glycoproteins need to be co-incorporated on the HCVpp to allow efficient virus entry and infection.

EXAMPLE 3 : HCVpp primary hepatocyte infectivity

5 Hepatocytes represent the principal site of HCV replication *in vivo*, yet *ex vivo* studies have suggested that HCV may also infect lymphoid cells (Lindenbach and Rice, 2001). To address whether either cell types could be infected, human adult hepatocytes (Figure 3) and peripheral blood mononuclear cells (PBMCs) have been transduced. Pseudo-particles were generated with core proteins derived from HIV-1
10 rather than from MLV, which do not permit transduction of non-proliferating target cells (Nègre *et al.*, 2000).

Cryopreserved primary hepatocytes, isolated from human adult biopsy samples checked for absence of HBV, HCV and HIV, were purchased from Biopredic International (Rennes, France) and cultured on collagen I coated plates according to
15 recommendations of the supplier. Transduction of hepatocytes was determined by counting GFP-positive cells under UV microscope. Specificity of infection was demonstrated by absence of transduction when target cells were infected with pseudo-particles lacking glycoproteins (pp cores) or by the reduced levels of transduction when target cells were pre-incubated with JS-81 anti-CD81 antibodies
20 (30 µg/ml) before infection. Infection of human PBMCs, isolated from healthy donors, was conducted as described previously (Sandrin *et al.*, 2002).

Relative to infection of Huh-7 cells, the HCVpp could readily infect the primary hepatocytes derived from different donors (Figure 3), yet transduction efficiencies varied with quality and cell culture viability of the individual biopsies. In contrast, poor
25 or undetectable infection of PBMCs was found with the HCVpp although control pseudo-particles generated with VSV-G could readily infect these primary cells.

Altogether these data indicate that HCVpp closely mimic the early events of infection by wild-type HCV and preferentially infect hepatocytes and hepatocarcinoma cells.

EXAMPLE 4 : **HCVpp is a valid model of early steps of HCV infection**

Infectivity of HCVpp is mediated by E1 and E2 glycoproteins

It was further determined whether infectivity of the HCVpp is specifically mediated by E1 and E2 and their interaction with cell surface receptors. A panel of monoclonal antibodies previously shown to specifically react against HCV-1a glycoproteins (Dubuisson *et al.*, 1994; Flint *et al.*, 1999; Patel *et al.*, 2000) was used.

HCVpp generated with HCV-1a E1E2 and with MLV core proteins were pre-incubated before infection of Huh-7 cells with saturating concentrations (20 µg/ml) of monoclonal antibodies against E1 (A4) or E2 (H31, H33, H35, H44, H48, H53, H54, H60 and H61) glycoproteins of genotype 1a, or with pooled antibodies (Hmix). Control experiments were performed using no antibodies or using pseudo-particles generated with VSV-G (VSV-Gpp).

Some of these monoclonal antibodies, like H35 and H48, for example, could neutralise the HCVpp of genotype 1a and reduce their infectivity by up to 70% (Figure 4). In contrast, none of these genotype 1a-specific antibodies could neutralise the HCVpp of genotype 1b or the control pseudo-particles generated with VSV-G (Figure 4). Neutralisation of the infectivity of these control pseudo-particles was achieved by using the VSV-G neutralising 41A.1 monoclonal antibody.

These data therefore demonstrate that infectivity of the pseudo-particles is specifically due to the incorporation of E1 and E2 glycoproteins, indicating that these HCVpp represent a valid model to investigate the early steps of HCV infection, *i.e.* receptor binding, membrane fusion and envelope uncoating.

HCV infection is neutralised by serum from HCV-infected patients

The capacity of sera derived from chronically HCV-infected patients to neutralise infectivity of HCVpp was further assessed.

HCVpp of genotypes 1a or 1b were pre-incubated for 30 min at room temperature with sera from chronically HCV-infected patients diluted 1/50 before infection of Huh-7 target cells. Control experiments were performed using pseudo-particles generated with RD114 glycoproteins (RD114pp), rather than with VSV-G that exhibits sensitivity to human complement (Sandrin *et al.*, 2002). Efficient neutralisation of the control pseudo-particles was demonstrated using a hyper-

immune goat serum raised against the RD114 SU glycoprotein (ViroMed Biosafety Laboratories, USA).

No or only weak neutralisation of control pseudo-particles could be detected using the sera of the HCV-infected patients. In contrast, most if not all of these sera could neutralise the infectivity of the HCVpp, in contrast to sera derived from healthy donors. Variable levels of neutralisation were detected depending on the donor and ranged from 20% to up to 90% of inhibition for HCVpp of both genotypes 1a and 1b. Sera derived from patients infected with HCV of the 1b genotype could neutralise the HCVpp generated with E1E2 of genotype 1a or 1b with similar efficiencies (Figure 5).

EXAMPLE 5 : Cell entry receptor usage by HCV pseudo-particles

Both the LDL receptor (LDLr) and CD81, a member of the tetraspanin family of receptors, have been proposed as putative HCV receptors (Pileri *et al.*, 1998; Agnello *et al.*, 1999). However, recent studies have questioned the role of these molecules as cell entry receptors despite their unequivocal capacity to bind HCV particles and/or glycoproteins (2023). Thus, the contribution of either cell surface molecules in the early stages of HCV cell entry was investigated by performing receptor-competition assays.

LDLr

LDLr binds apolipoprotein B within LDL and apolipoproteins B and E (ApoB and ApoE) within VLDL complexes; both complexes have been found associated with HCV particles in plasma of infected patients (Andre *et al.*, 2002).

LDL receptor competition assays were performed using purified LDLs (10 µg/ml, 100 µg/ml; Sigma-Aldrich, France) or with the monoclonal antibodies 5E11 (10 µg/ml, 50 µg/ml), 4G3 (anti-ApoB; 10 µg/ml, 50 µg/ml) or 1B7 (anti-ApoE; 1 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml) (Ottawa Heart Institute Research corporation, Ottawa, Ontario, Canada), or mixed 5E11, 4G3 and 1B7 antibodies (with individual concentrations 10 µg/ml, 50 µg/ml), pre-incubated with the pseudo-particles prior to infection.

Purified LDLs were not found to out-compete infection of HCVpp on Huh-7 LDLr-positive cells, even when concentrations higher than 100 µg/ml were used.

When monoclonal antibodies targeted to the receptor binding sites of ApoB and ApoE were pre-incubated with the HCVpp, only the anti-ApoE antibodies could

inhibit, albeit weakly, the infectivity of the HCVpp. This is consistent with our observation that HCVpp could not or hardly infect LDLr-positive cell lines such as HepG2, Jurkat, CEM and TE671 (Figure 1). Thus, although these results do not exclude a role of LDLr in HCV entry, possibly *via* association of LDLs with HCV particles (Andre *et al.*, 2002), they suggest that LDLr is not the major receptor of HCVpp entry.

CD81

Recombinant GST-fusion polypeptides encompassing the large extra-cellular loop (LEL) of human CD81 which has been shown to bind HCV E2 (Flint *et al.*, 1999, (Pileri *et al.*, 1998) were then used to investigate the role of hCD81 in HCVpp cell entry.

hCD81-LEL GST-fusion polypeptides (4 µg/ml, 8 µg/ml, 16 µg/ml) were pre-incubated with the pseudo-particles before infection or JS-81 anti-CD81 antibodies (4 µg/ml, 10 µg/ml, 30 µg/ml; Pharmingen, France) were pre-incubated with the target cells prior to infection. Percentages of inhibition of the infectious titres obtained on Huh-7 cells relative to titres obtained in the absence of inhibitors were calculated. Control experiments were performed using pseudo-particles generated with VSV-G (VSV-Gpp). Comparative experiments were further conducted with NIH3T3, NIH3T3-hCD81 and Huh-7 cells infected with HCVpp and with control pseudo-particles generated with VSV-G.

The GST-fusion hCD81-LEL polypeptides pre-incubated with the HCVpp could specifically precipitate E1E2 complexes, confirming their capacity to bind the E2 glycoprotein (Flint *et al.*, 1999; Pileri *et al.*, 1998), and were found to neutralise the infectivity of the HCVpp on Huh-7 cells, yet with a relatively poor efficiency. Inhibition ranged from 38% to 54% inhibition of infectivity and appeared to be dose-dependent.

Consistently, pre-incubation of Huh-7 cells with JS-81 monoclonal antibody, that blocks binding of recombinant E2 to hCD81 (Flint *et al.*, 1999), was found to inhibit infectivity of HCVpp in both Huh-7 target cells and human primary hepatocytes. At high antibody concentrations (30 µg/ml), over 90% inhibition of infection could be obtained for HCVpp based on genotypes 1a as well as 1b. However, several target cells non-permissive to infection by HCVpp, like TE671, Jurkat, and CEM cells (Figure 1) were found to express high densities of hCD81, indicating the lack of correlation between CD81 expression and infectivity.

Moreover, expression of hCD81 in non-permissive NIH3T3 mouse fibroblasts did not allow infection with the HCVpp (Figure 4C).

Altogether these results demonstrate that although hCD81 binds HCV E2 and might help cell surface attachment of HCV, it is not sufficient by itself to allow infection with HCVpp.

EXAMPLE 6 : Deletion of hypervariable region I

HCV infection is not cleared by over 80% of patients and chronicity is associated with various forms of liver disease. Chronic infection is thought to be caused by the high mutation rate of HCV, which helps the virus to escape the host immune response. One of several mutation hotspots within the HCV genome has been localised to the N-terminus of the E2 glycoprotein. This so-called hypervariable region I is a major antigen and is thought to play an important role in allowing HCV to escape the host's immune response. The antigenic variation of the HVR1 also inhibits the development of a protective immune response against re-infection with heterologous HCV strains/genotypes. Finally does the antigenic dominance and extreme variability of the HVR1 hinder the development of vaccines against HCV. With the long-term aim to develop effective therapeutics which target more conserved parts of the HCV glycoproteins the inventors investigated whether the HVR1 is dispensable for infection.

For that purpose expression constructs encoding the polyprotein for wildtype E1E2 or a mutant version in which the HVR1 = the first 27 residues of E2 had been deleted from the polyprotein were generated. HCV pseudo-particles (HCVpp) were produced with either wildtype or Δ HVR1 Δ CE1E2 polyprotein. Analysis of immunoblots of transfected cells showed that both wildtype and mutant E2 proteins were expressed to similar levels. Viral particles were harvested from the supernatant of transfected cells and purified by ultra-centrifugation through high-density sucrose cushions. Δ HVR1 and wildtype E2 were incorporated into particles to a similar level (Fig. 7). The infectivity of the wildtype and mutant HCVpp were tested as before on Huh-7 target cells. Infectious titres of up to 2.6×10^5 TU/ml were detected for wildtype HCVpp (Fig. 8). Δ HVR1 HCVpp were about 5 to 10 fold less infectious, still a maximal titer of 5.1×10^5 TU/ml was observed suggesting that the HVR1 aids but is not required for the infection process.

In conclusion, the invention provides a tool that allows precise investigation of viral assembly of E1E2 glycoproteins (processing, maturation) and their role in cell entry of HCV. No structural modifications of the E1E2 glycoproteins were required for their correct assembly on retroviral and lentiviral cores and to generate high titre infectious HCVpp with functional E1E2 glycoproteins. The insertion of a marker gene into the HCVpp allowed precise and rapid determination of the infectivity of these pseudo-particles by flow-cytometry.

The development of these functional, infectious HCV pseudo-particles make it now possible to investigate early events of HCV infection, such as the identification of novel HCV receptor(s) or co-receptor(s), to carry out diagnostic assays for the detection of neutralising antibodies in seroconverted patients, and to develop efficient inhibitors to HCV infection.

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CLAIMS

1. A method for producing hepatitis C virus (HCV)-like particles *ex vivo*
5 comprising the steps of:
 - providing a first nucleic acid sequence comprising a packaging competent retroviral-derived genome;
 - providing a second nucleic acid sequence comprising a cDNA encoding core
10 proteins from said retrovirus;
 - providing a third nucleic acid sequence comprising a cDNA encoding a polyprotein comprising successively a HCV core protein, and a HCV E1 protein and/or a HCV E2 protein;
 - transfecting host cells with said nucleic acid sequences and maintaining the
15 transfected cells in culture for sufficient time to allow expression of the cDNAs to produce structural proteins from hepatitis C virus and retrovirus; and allowing the structural proteins to form virus-like particles.
2. The method according to claim 1, wherein said packaging competent retroviral-derived genome and core proteins are from a retrovirus selected from the group consisting of MLV, ALV, RSV, MPMV, HIV-1, HIV-2, SIV, EIAV, CAEV, or
20 HFV.
3. The method according to claim 1 or 2, wherein said HCV core protein comprises the last 21 amino acids of the carboxy-terminus of HCV core
4. The method according to any of claims 1 to 3, wherein said polyprotein comprises a HCV core protein and a HCV E1 protein.
- 25 5. The method according to any of claims 1 to 3, wherein said polyprotein comprises a HCV core protein and a HCV E2 protein.
6. The method according to any of claims 1 to 5, comprising native HCV E1 and/or E2 protein.
7. The method according to any of claims 1 to 3, wherein said polyprotein
30 comprises a HCV core protein, native HCV E1 protein and native HCV E2 protein.
8. The method according to any of preceding claims, wherein said nucleic acid sequence comprising a packaging competent retroviral-derived genome further comprises a transgene.

9. An infectious HCV-like particle susceptible to be obtained by a method according to any of preceding claims, comprising the core proteins from a retrovirus, and a E1 HCV glycoprotein and/or a E2 HCV glycoprotein.

10. The infectious particle according to claim 9, comprising E1 and E2 HCV glycoproteins.

11. The infectious particle according to claim 9, comprising E1 HCV glycoprotein.

12. The infectious particle according to claim 9, comprising E2 HCV glycoprotein.

13. The infectious particle according to any of claims 9 to 12, comprising native E1 and/or E2 HCV glycoprotein.

14. The infectious particle according to any of claims 9 to 13, wherein said retrovirus is selected from the group consisting of MLV, ALV, RSV, MPMV, HIV-1, HIV-2, SIV, EIAV, CAEV, or HFV.

15. The infectious particle according to any of claims 9 to 14, wherein said nucleic acid sequence comprising a packaging competent retroviral-derived genome further comprises a transgene.

16. Use of three nucleic acid sequences for the preparation of a medicament useful as a vaccine against hepatitis C, wherein the nucleic acid sequences are :

- a first nucleic acid sequence comprising a packaging competent retroviral-derived genome;

- a second nucleic acid sequence comprising a cDNA encoding core proteins from said retrovirus;

- a third nucleic acid sequence comprising a cDNA encoding a polyprotein comprising successively a HCV core protein, and a HCV E1 protein and/or a HCV E2 protein ;

and, when transferred into cells of a subject, the nucleic acids sequences allow the production of structural proteins from hepatitis C virus and retrovirus, wherein the structural proteins form virus-like particles that are immunogenic.

17. A method for *ex vivo* identification of a receptor for HCV E1 and/or E2 glycoprotein comprising detection of the binding of an infectious particle according to any of claims 9 to 14, to a cell receptor.

18. A method for *ex vivo* identifying a cell receptor for HCV comprising the step consisting of:

- transfecting a cell which is not permissive for HCV infection with a nucleic acid sequence encoding a protein likely to be a receptor for HCV;

- contacting said transformed cell with a HCV-like particle according to any of claims 9 to 14;

- determining whether said transformed cell has become permissive or not for HCV infection; and

- identifying as a cell receptor for HCV said protein expressed by the transformed cell that has become permissive.

19. A method for *ex vivo* identifying a cell receptor for HCV comprising the step consisting of:

- providing an expression cDNA library obtained from a cell permissive for HCV infection;

- transfecting cells that are not permissive for HCV infection with said expression cDNA library;

- contacting said transformed cells with HCV-like particles according to any of claims 9 to 14;

- identifying and isolating those transformed cells that have become permissive for HCV infection;

- isolating the expression vector transfected in cells that have become permissive; and

- identifying as a receptor for HCV the proteins encoded by the cDNA sequence of said isolated expression vectors.

20. A method of *ex vivo* screening or identification of molecules capable of interfering with HCV entry in cells comprising comparison of the level of cell infection by an infectious particle according to any of claims 9 to 14 in the presence or the absence of a candidate molecule.

21. A method of *in vitro* diagnosis of a HCV infection in a patient, comprising detecting immune complexes formed by interaction of anti-HCV antibodies likely to be present in a biological sample of the patient with HCV-like particle according to any of claims 9 to 14.

22. A method of *in vitro* diagnosis of a HCV infection in a patient, comprising detecting an inhibitory effect of anti-HCV antibodies likely to be present in

a biological sample of the patient, on the infection of a permissive cell by HCV-like particles according to any of claims 9 to 14.

23. A diagnostic kit useful for the method of claim 21, comprising a HCV-like particle according to any of claim 9 to 14 and appropriate means of detection of
5 said immune complexes.

24. Vaccine composition comprising a HCV-like particle according to any of claims 9 to 15 and a pharmaceutically acceptable carrier.

25. A method for *in vitro* transferring a transgene of interest in a hepatic cell comprising infecting a cell with a HCV-like particle as described in any of claims 9 to
10 14, wherein the HCV-like particle carries a transgene of interest.

26. Use of a HCV-like particle according to any of claims 9 to 14, that carries a transgene of interest, for the preparation of a medicament for the prevention or treatment of a disease in a patient, wherein the HCV-like particle allows the transfer of the transgene of interest into a cell of the patient, and encodes a product
15 that has a prophylactic or therapeutic effect against the disease.

**INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE
(INSERM)**

**Infectious HCV pseudo-particles containing functional
E1, E2 envelope proteins**

Abstract

The invention relates to the generation and the use of HCV pseudo-particles containing native functional E1, E2 envelope glycoproteins assembled onto retroviral core particles. These particles are highly infectious and constitute a valid model of HCV virion.

1/6

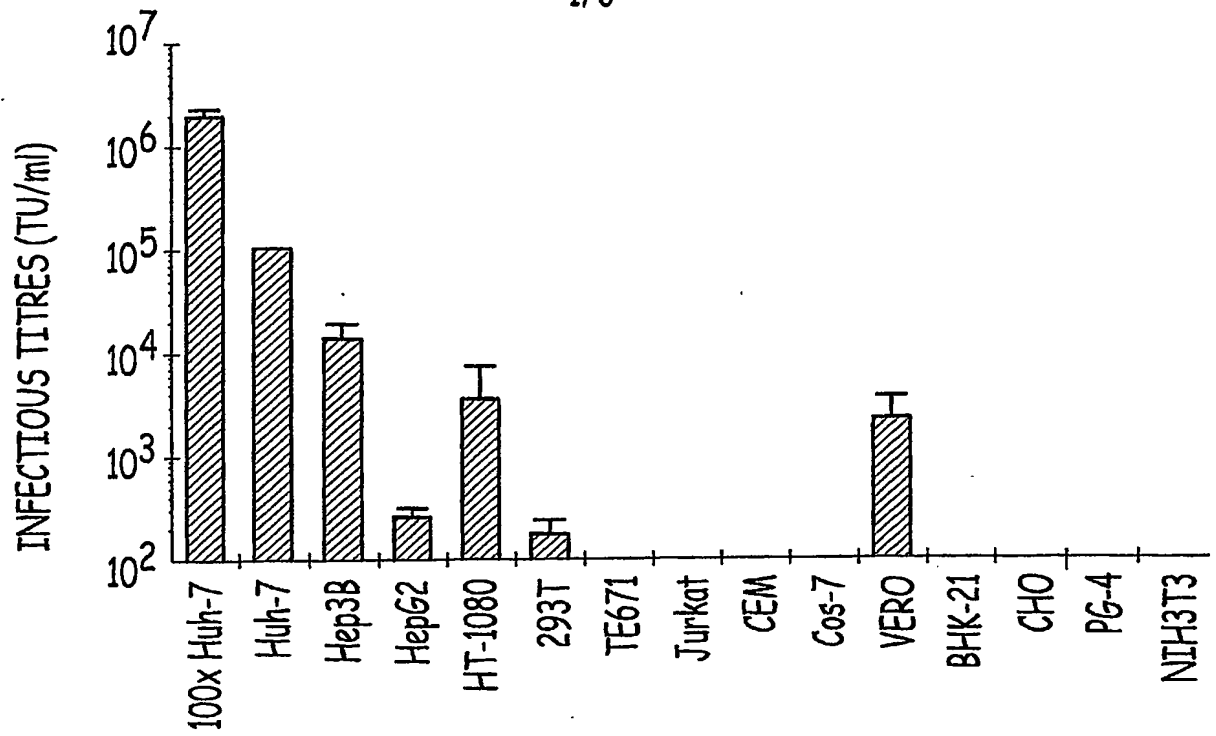


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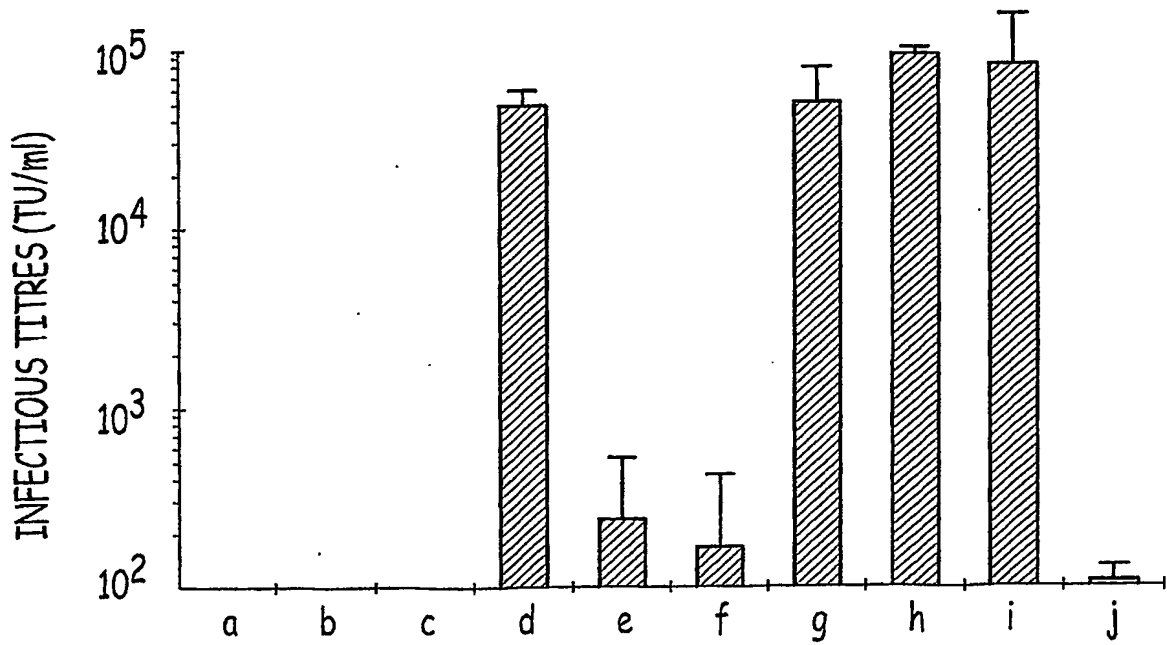


FIG.2

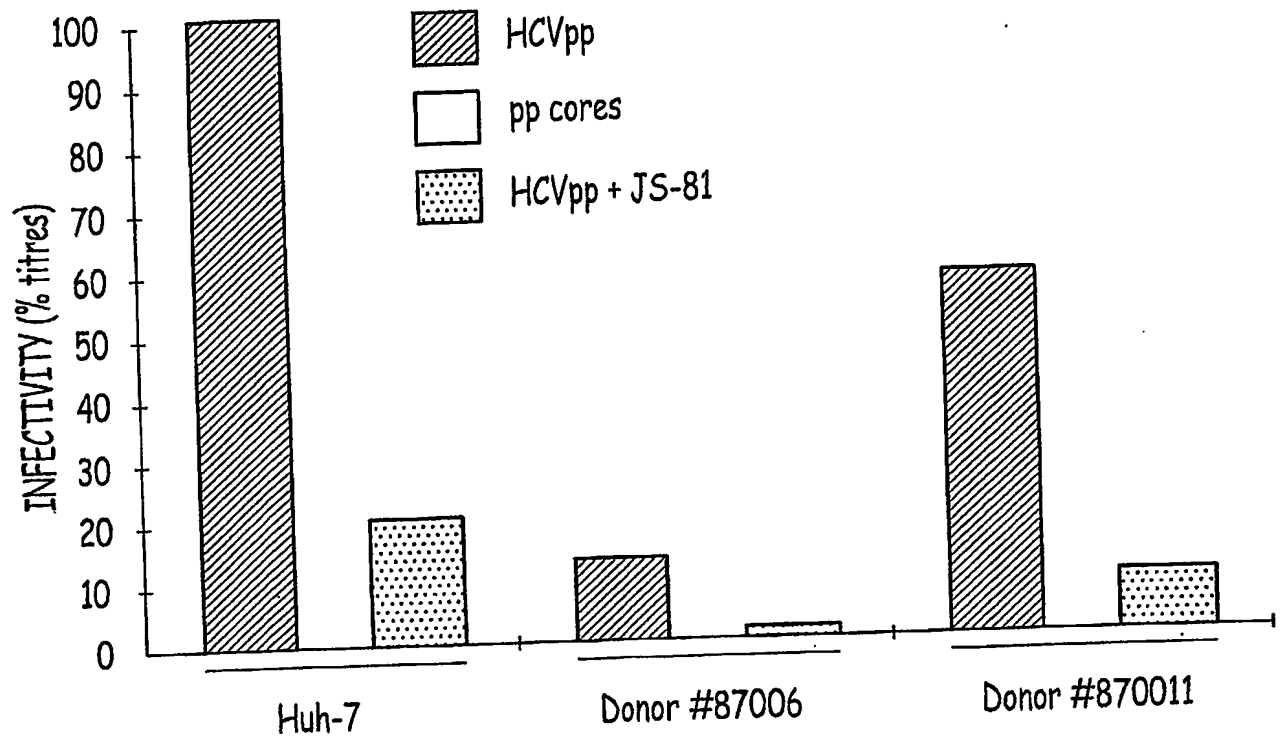
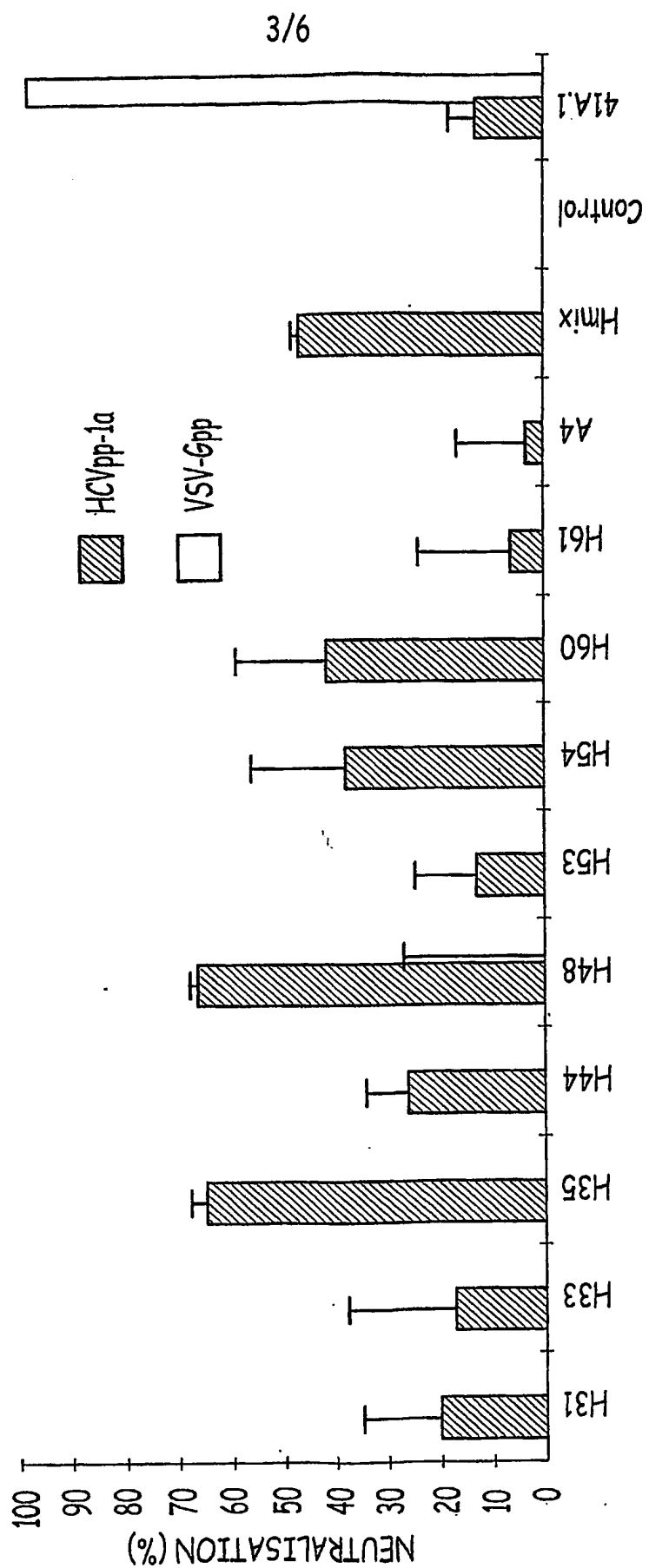
FIG.3

FIG. 4



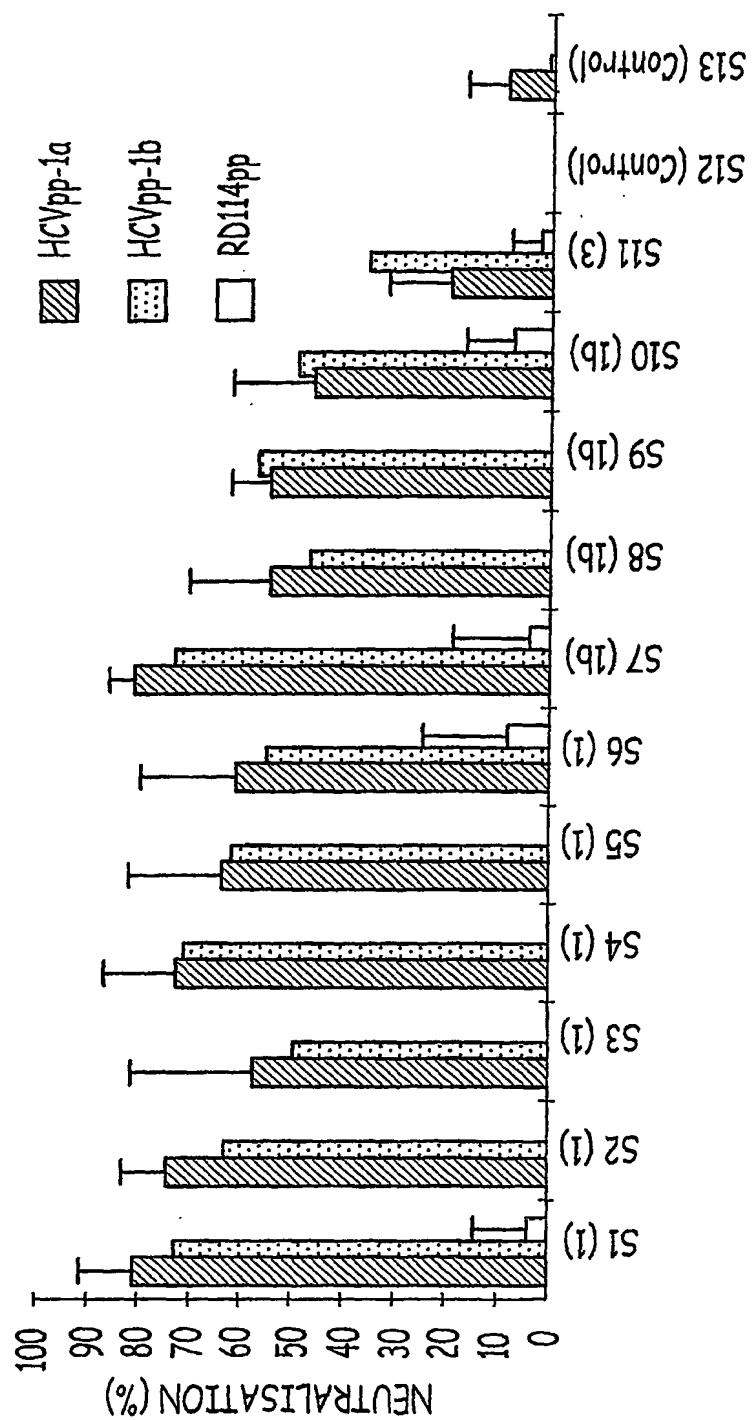
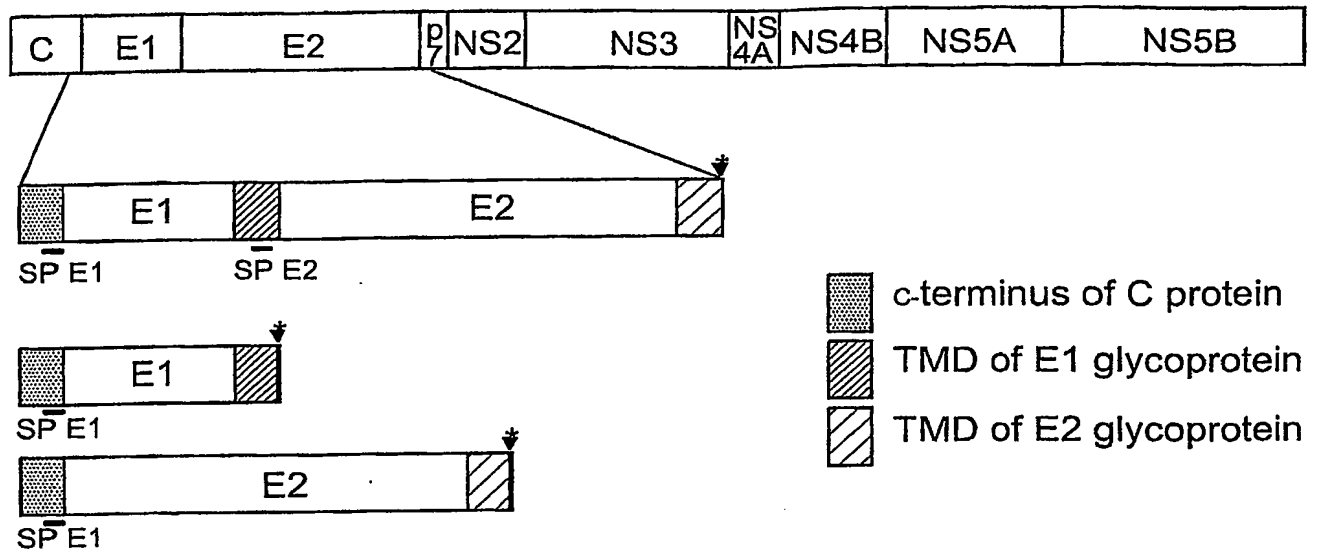
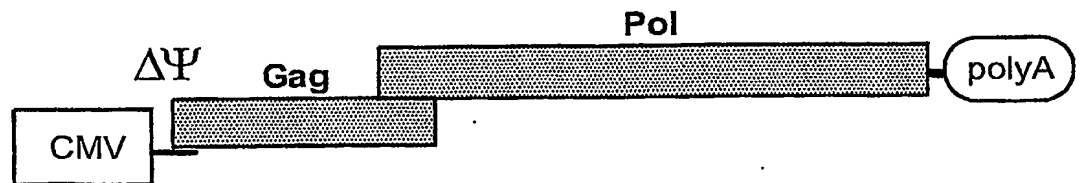


FIG.5

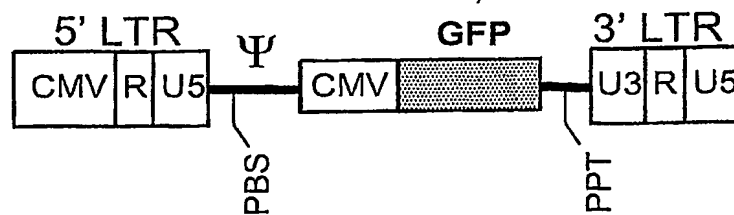
**FIG.6A**

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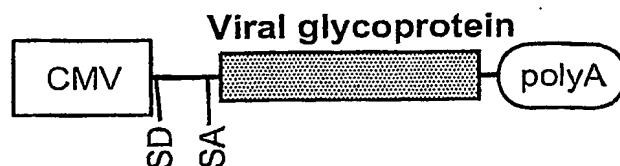
Retroviral core proteins

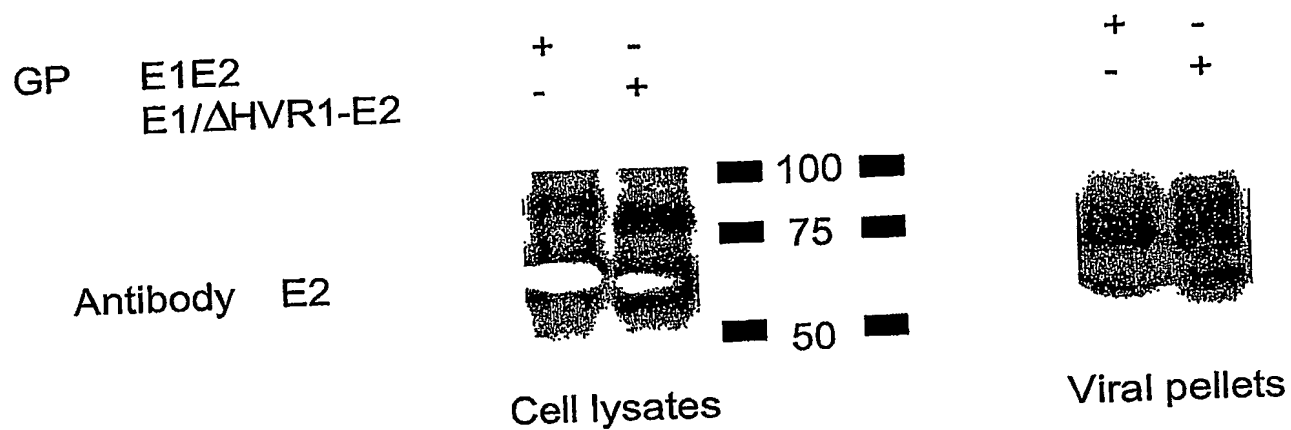
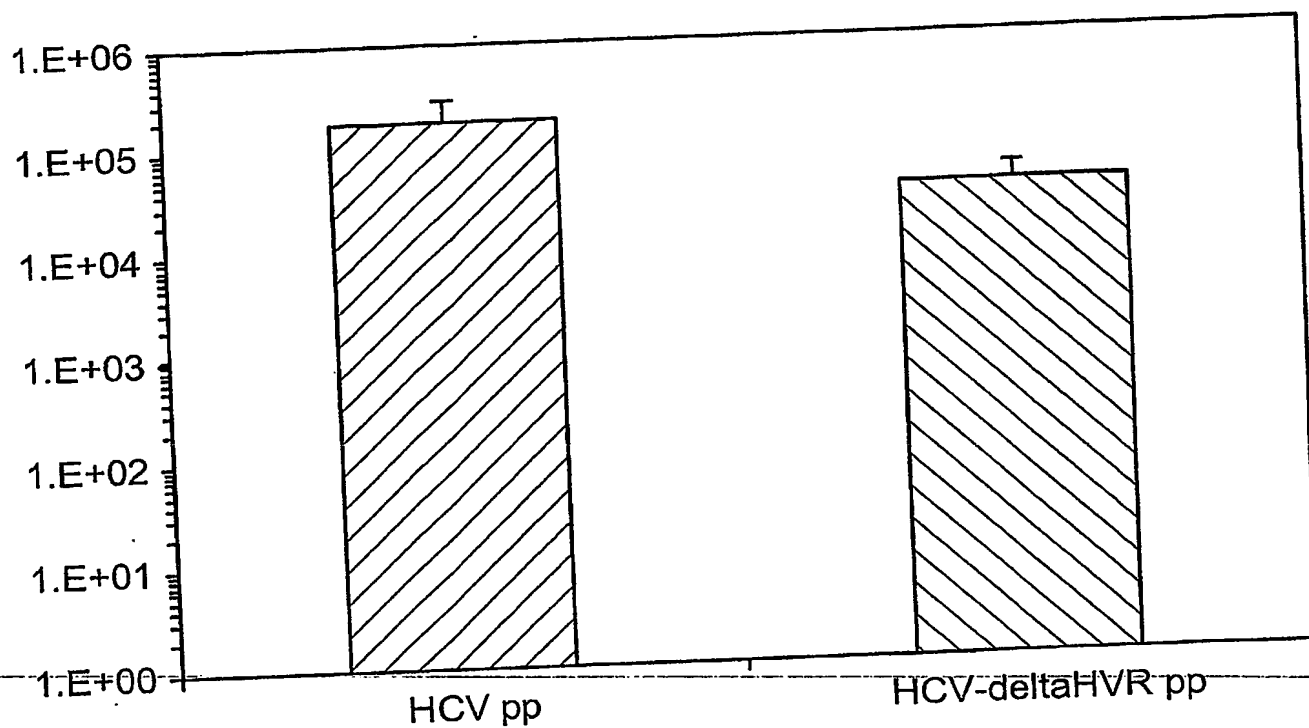


GFP transfer vector



Envelope glycoproteins

**FIG.6B**

FIG.7FIG.8

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 in BamHI-blunted of hCMV-G

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<211> 617

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence:
phCMV-deltaCE1E2-1a plasmid

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Phe	Leu	Leu	Ala	Leu	Leu	Ser	Cys	Leu	Thr	Val	Pro	Ala	Ser	Ala	Tyr
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Gly	Cys	Val	Pro	Cys	Val	Arg	Glu	Gly	Asn	Ala	Ser	Arg	Cys	Trp	Val
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Gln	Leu	Arg	Arg	His	Ile	Asp	Leu	Leu	Val	Gly	Ser	Ala	Thr	Leu	Cys
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Gln	Leu	Phe	Thr	Phe	Ser	Pro	Arg	Arg	His	Trp	Thr	Thr	Gln	Ser	Cys
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Asn Cys Ser Ile Tyr Pro Gly His Ile Thr Gly His Arg Met Ala Trp
180 185 190

Asp Met Met Met Asn Trp Ser Pro Thr Ala Ala Leu Val Val Ala Gln
195 200 205

Leu Leu Arg Ile Pro Gln Ala Ile Met Asp Met Ile Ala Gly Ala His
210 215 220

Trp Gly Val Leu Ala Gly Ile Ala Tyr Phe Ser Met Val Gly Asn Trp
225 230 235 240

Ala Lys Val Leu Val Val Leu Leu Leu Phe Ala Gly Val Asp Ala Glu
245 250 255

Thr His Val Thr Gly Gly Ser Ala Gly His Thr Thr Ala Gly Leu Val
260 265 270

Gly Leu Leu Thr Pro Gly Ala Lys Gln Asn Ile Gln Leu Ile Asn Thr
275 280 285

Asn Gly Ser Trp His Ile Asn Ser Thr Ala Leu Asn Cys Asn Asp Ser
290 295 300

Leu Thr Thr Gly Trp Leu Ala Gly Leu Phe Tyr Arg His Lys Phe Asn
305 310 315 320

Ser Ser Gly Cys Pro Glu Arg Leu Ala Ser Cys Arg Arg Leu Thr Asp
325 330 335

Phe Ala Gln Gly Trp Gly Pro Ile Ser Tyr Ala Asn Gly Ser Gly Leu
340 345 350

Asp Glu Arg Pro Tyr Cys Trp His Tyr Pro Pro Arg Pro Cys Gly Ile
355 360 365

Val Pro Ala Lys Ser Val Cys Gly Pro Val Tyr Cys Phe Thr Pro Ser
370 375 380

Pro Val Val Val Gly Thr Thr Asp Arg Ser Gly Ala Pro Thr Tyr Ser
385 390 395 400

Trp Gly Ala Asn Asp Thr Asp Val Phe Val Leu Asn Asn Thr Arg Pro
405 410 415

Pro Leu Gly Asn Trp Phe Gly Cys Thr Trp Met Asn Ser Thr Gly Phe
420 425 430

Thr Lys Val Cys Gly Ala Pro Pro Cys Val Ile Gly Gly Val Gly Asn
435 440 445

Asn Thr Leu Leu Cys Pro Thr Asp Cys Phe Arg Lys His Pro Glu Ala
450 455 460

Thr Tyr Ser Arg Cys Gly Ser Gly Pro Trp Ile Thr Pro Arg Cys Met
465 470 475 480

Val Asp Tyr Pro Tyr Arg Leu Trp His Tyr Pro Cys Thr Ile Asn Tyr
485 490 495

Thr Ile Phe Lys Val Arg Met Tyr Val Gly Gly Val Glu His Arg Leu

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Arg Asp Arg Ser Glu Leu Ser Pro Leu Leu Leu Ser Thr Thr Gln Trp 530 535 540		
Gln Val Leu Pro Cys Ser Phe Thr Thr Leu Pro Ala Leu Ser Thr Gly 545 550 555 560		
Leu Ile His Leu His Gln Asn Ile Val Asp Val Gln Tyr Leu Tyr Gly 565 570 575		
Val Gly Ser Ser Ile Ala Ser Trp Ala Ile Lys Trp Glu Tyr Val Val 580 585 590		
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 <211> 7178
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:
 pHCMV-deltaCE2-1a plasmid

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<220>
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 <222> (769)..(1429)
 <223> rabbit beta-globin intron II

<220>
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 <222> (1425)..(1430)
 <223> BamHI 5' junction after ligation of deltaCE2
 fragment in BamHI site of hCMV-G

<220>
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 <222> (1430)..(2115)
 <223> IRES sequence of EMCV

<220>
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<220>
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 <222> (2125)..(2304)
 <223> deltaC part of the polyprotein

<220>
 <221> misc_feature
 <222> (2305)..(3390)
 <223> Mature E2 protein

<220>
 <221> misc_feature
 <222> (3403)..(3778)
 <223> Sinbis Virus junk sequence

<220>
 <221> misc_feature
 <222> (3787)..(3792)
 <223> BamHI 3' junction after ligation of deltaCE1
 fragment in BamHI site of HCMV-G

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 Val Leu Glu Asp Gly Val Asn Tyr Ala Thr Gly Asn Leu Pro Gly Cys
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 Phe Phe Ser Ile Phe Leu Leu Ala Leu Leu Ser Cys Leu Thr Val Pro
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 Ala Ser Ala Glu Thr His Val Thr Gly Gly Ser Ala Gly His Thr Thr
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 95 100 105
 tgc aac gat agc ctt acc acc ggc tgg tta gca ggg ctc ttc tat cgc 2487
 Cys Asn Asp Ser Leu Thr Thr Gly Trp Leu Ala Gly Leu Phe Tyr Arg
 110 115 120

cac aaa ttc aac tct tca ggc tgt cct gag agg ttg gcc agc tgc cga 2535
 His Lys Phe Asn Ser Ser Gly Cys Pro Glu Arg Leu Ala Ser Cys Arg
 125 130 135 140

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 Arg Leu Thr Asp Phe Ala Gln Gly Trp Gly Pro Ile Ser Tyr Ala Asn
 145 150 155

gga agc ggc ctt gac gaa cgc ccc tac tgt tgg cac tac cct cca aga 2631
 Gly Ser Gly Leu Asp Glu Arg Pro Tyr Cys Trp His Tyr Pro Pro Arg
 160 165 170

cct tgt ggc att gtg ccc gca aag agc gtg tgt ggc ccg gta tat tgc 2679
 Pro Cys Gly Ile Val Pro Ala Lys Ser Val Cys Gly Pro Val Tyr Cys
 175 180 185

ttc act ccc agc ccc gtg gtg gtg gga acg acc gac agg tcg ggc gcg 2727
 Phe Thr Pro Ser Pro Val Val Val Gly Thr Thr Asp Arg Ser Gly Ala
 190 195 200

cct acc tac agc tgg ggt gca aat gat acg gat gtc ttc gtc ctt aac 2775
 Pro Thr Tyr Ser Trp Gly Ala Asn Asp Thr Asp Val Phe Val Leu Asn
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aac acc agg cca ccg ctg ggc aat tgg ttc ggt tgt acc tgg atg aac 2823
 Asn Thr Arg Pro Leu Gly Asn Trp Phe Gly Cys Thr Trp Met Asn
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tca act gga ttc acc aaa gtg tgc gga gcg ccc cct tgt gtc atc gga 2871
 Ser Thr Gly Phe Thr Lys Val Cys Gly Ala Pro Pro Cys Val Ile Gly
 240 245 250

ggg gtg ggc aac aac acc ttg ctc tgc ccc act gat tgc ttc cgc aaa 2919
 Gly Val Gly Asn Asn Thr Leu Leu Cys Pro Thr Asp Cys Phe Arg Lys
 255 260 265

cat ccg gaa gcc aca tac tct cgg tgc ggc tcc ggt ccc tgg att aca 2967
 His Pro Glu Ala Thr Tyr Ser Arg Cys Gly Ser Gly Pro Trp Ile Thr
 270 275 280

ccc agg tgc atg gtc gac tac ccg tat agg ctt tgg cac tat cct tgt 3015
 Pro Arg Cys Met Val Asp Tyr Pro Tyr Arg Leu Trp His Tyr Pro Cys
 285 290 295 300

act atc aat tac acc ata ttc aaa gtc agg atg tac gtg gga ggg gtc 3063
 Thr Ile Asn Tyr Thr Ile Phe Lys Val Arg Met Tyr Val Gly Gly Val
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gag cac agg ctg gaa gcg gcc tgc aac tgg acg cgg ggc gaa cgc tgt 3111
 Glu His Arg Leu Glu Ala Ala Cys Asn Trp Thr Arg Gly Glu Arg Cys
 320 325 330

gat ctg gaa gac agg gac agg tcc gag ctc agc cca ttg ctg ctg tcc 3159
 Asp Leu Glu Asp Arg Asp Arg Ser Glu Leu Ser Pro Leu Leu Leu Ser
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acc aca cag tgg cag gtc ctt ccg tgt tct ttc acg acc ctg cca gcc 3207
 Thr Thr Gln Trp Gln Val Leu Pro Cys Ser Phe Thr Thr Leu Pro Ala
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<210> 13

<211> 425

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence:

phCMV-deltaCE2-1a plasmid

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Phe	Leu	Leu	Ala	Leu	Leu	Ser	Cys	Leu	Thr	Val	Pro	Ala	Ser	Ala	Glu
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Gly	Leu	Leu	Thr	Pro	Gly	Ala	Lys	Gln	Asn	Ile	Gln	Leu	Ile	Asn	Thr
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Leu	Thr	Thr	Gly	Trp	Leu	Ala	Gly	Leu	Phe	Tyr	Arg	His	Lys	Phe	Asn
		115					120					125			
Ser	Ser	Gly	Cys	Pro	Glu	Arg	Leu	Ala	Ser	Cys	Arg	Arg	Leu	Thr	Asp
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Phe	Ala	Gln	Gly	Trp	Gly	Pro	Ile	Ser	Tyr	Ala	Asn	Gly	Ser	Gly	Leu
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Val Pro Ala Lys Ser Val Cys Gly Pro Val Tyr Cys Phe Thr Pro Ser
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Pro Val Val Val Gly Thr Thr Asp Arg Ser Gly Ala Pro Thr Tyr Ser
 195 200 205

Trp Gly Ala Asn Asp Thr Asp Val Phe Val Leu Asn Asn Thr Arg Pro
 210 215 220

Pro Leu Gly Asn Trp Phe Gly Cys Thr Trp Met Asn Ser Thr Gly Phe
 225 230 235 240

Thr Lys Val Cys Gly Ala Pro Pro Cys Val Ile Gly Gly Val Gly Asn
 245 250 255

Asn Thr Leu Leu Cys Pro Thr Asp Cys Phe Arg Lys His Pro Glu Ala
 260 265 270

Thr Tyr Ser Arg Cys Gly Ser Gly Pro Trp Ile Thr Pro Arg Cys Met
 275 280 285

Val Asp Tyr Pro Tyr Arg Leu Trp His Tyr Pro Cys Thr Ile Asn Tyr
 290 295 300

Thr Ile Phe Lys Val Arg Met Tyr Val Gly Gly Val Glu His Arg Leu
 305 310 315 320

Glu Ala Ala Cys Asn Trp Thr Arg Gly Glu Arg Cys Asp Leu Glu Asp
 325 330 335

Arg Asp Arg Ser Glu Leu Ser Pro Leu Leu Leu Ser Thr Thr Gln Trp
 340 345 350

Gln Val Leu Pro Cys Ser Phe Thr Thr Leu Pro Ala Leu Ser Thr Gly
 355 360 365

Leu Ile His Leu His Gln Asn Ile Val Asp Val Gln Tyr Leu Tyr Gly
 370 375 380

Val Gly Ser Ser Ile Ala Ser Trp Ala Ile Lys Trp Glu Tyr Val Val
 385 390 395 400

Leu Leu Phe Leu Leu Leu Ala Asp Ala Arg Val Cys Ser Cys Leu Trp
 405 410 415

Met Met Leu Leu Ile Ser Gln Ala Glu
 420 425

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